A

Practitioner's Docket No. NEB-154 **PATENT**

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Box Patent Application
Assistant Commissioner for Patents
Washington, D.C. 20231

NEW APPLICATION TRANSMITTAL

Transmitted herewith for filing is the patent application of

Inventor(s): Thomas C. EVANS
Ming-Qun XU

WARNING: *37 C.F.R. § 1.41(a)(1) points out:*

"(a) A patent is applied for in the name or names of the actual inventor or inventors.

"(1) The inventorship of a nonprovisional application is that inventorship set forth in the oath or declaration as prescribed by § 1.63, except as provided for in § 1.53(d)(4) and § 1.63(d). If an oath or declaration as prescribed by § 1.63 is not filed during the pendency of a nonprovisional application, the inventorship is that inventorship set forth in the application papers filed pursuant to § 1.53(b), unless a petition under this paragraph accompanied by the fee set forth in § 1.17(i) is filed supplying or changing the name or names of the inventor or inventors."

For (title):
INTEIN-MEDIATED PROTEIN LIGATION OF EXPRESSED PROTEINS

CERTIFICATION UNDER 37 C.F.R. § 1.10*
(Express Mail label number is mandatory.)
(Express Mail certification is optional.)

I hereby certify that this New Application Transmittal and the documents referred to as attached therein are being deposited with the United States Postal Service on this date 12 February 1999, in an envelope as "Express Mail Post Office to Addressee," mailing Label Number EE466580584US, addressed to the: Assistant Commissioner for Patents, Washington, D.C. 20231.

Melissa A. Schicking
(type or print name of person mailing paper)

Melissa A. Schicking
Signature of person mailing paper

WARNING: *Certificate of mailing (first class) or facsimile transmission procedures of 37 C.F.R. § 1.8 cannot be used to obtain a date of mailing or transmission for this correspondence.*

***WARNING:** Each paper or fee filed by "Express Mail" **must** have the number of the "Express Mail" mailing label placed thereon prior to mailing. 37 C.F.R. § 1.10(b).*
*"Since the filing of correspondence under § 1.10 without the Express Mail mailing label thereon is an oversight that can be avoided by the exercise of reasonable care, requests for waiver of this requirement will **not** be granted on petition." Notice of Oct. 24, 1996, 60 Fed. Reg. 56,439, at 56,442.*

1. Type of Application

This new application is for a(n)

(check one applicable item below)

☒ Original (nonprovisional)

☐ Design

☐ Plant

WARNING: ***Do not*** *use this transmittal for a completion in the U.S. of an International Application under 35 U.S.C. § 371(c)(4), unless the International Application is being filed as a divisional, continuation or continuation-in-part application.*

WARNING: ***Do not*** *use this transmittal for the filing of a provisional application.*

NOTE: If one of the following 3 items apply, then complete and attach ADDED PAGES FOR NEW APPLICATION TRANSMITTAL WHERE BENEFIT OF A PRIOR U.S. APPLICATION CLAIMED and a NOTIFICATION IN PARENT APPLICATION OF THE FILING OF THIS CONTINUATION APPLICATION.

☐ Divisional.

☐ Continuation.

☐ Continuation-in-part (C-I-P).

2. Benefit of Prior U.S. Application(s) (35 U.S.C. §§ 119(e), 120, or 121)

NOTE: A nonprovisional application may claim an invention disclosed in one or more prior filed copending nonprovisional applications or copending international applications designating the United States of America. In order for a nonprovisional application to claim the benefit of a prior filed copending nonprovisional application or copending international application designating the United States of America, each prior application must name as an inventor at least one inventor named in the later filed nonprovisional application and disclose the named inventor's invention claimed in at least one claim of the later filed nonprovisional application in the manner provided by the first paragraph of 35 U.S.C. § 112. Each prior application must also be:

(i) An international application entitled to a filing date in accordance with PCT Article 11 and designating the United States of America; or

(ii) Complete as set forth in § 1.51(b); or

(iii) Entitled to a filing date as set forth in § 1.53(b) or § 1.53(d) and include the basic filing fee set forth in § 1.16; or

(iv) Entitled to a filing date as set forth in § 1.53(b) and have paid therein the processing and retention fee set forth in § 1.21(l) within the time period set forth in § 1.53(f).

37 C.F.R. § 1.78(a)(1).

NOTE: If the new application being transmitted is a divisional, continuation or a continuation-in-part of a parent case, or where the parent case is an International Application which designated the U.S., or benefit of a prior provisional application is claimed, then check the following item and complete and attach ADDED PAGES FOR NEW APPLICATION TRANSMITTAL WHERE BENEFIT OF PRIOR U.S. APPLICATION(S) CLAIMED.

WARNING: *If an application claims the benefit of the filing date of an earlier filed application under 35 U.S.C. §§ 120, 121 or 365(c), the 20-year term of that application will be based upon the filing date of the earliest U.S. application that the application makes reference to under 35 U.S.C. §§ 120, 121 or 365(c). (35 U.S.C. § 154(a)(2) does not take into account, for the determination of the patent term, any application on which priority is claimed under 35 U.S.C. §§ 119, 365(a) or 365(b).) For a c-i-p application, applicant should review whether any claim in the patent that will issue is supported by an earlier application and, if not, the applicant should consider canceling the reference to the earlier filed application. The term of a patent is not based on a claim-by-claim approach. See Notice of April 14, 1995, 60 Fed. Reg. 20,195, at 20,205.*

WARNING: *When the last day of pendency of a provisional application falls on a Saturday, Sunday, or Federal holiday within the District of Columbia, any nonprovisional application claiming benefit of the provisional application* ***must*** *be filed prior to the Saturday, Sunday, or Federal holiday within the District of Columbia. See 37 C.F.R. § 1.78(a)(3).*

☒ The new application being transmitted claims the benefit of prior U.S. application(s). Enclosed are ADDED PAGES FOR NEW APPLICATION TRANSMITTAL WHERE BENEFIT OF PRIOR U.S. APPLICATION(S) CLAIMED.

3. Papers Enclosed

A. Required for filing date under 37 C.F.R. § 1.53(b) (Regular) or 37 C.F.R. § 1.153 (Design) Application

22 Pages of specification (includes cover page)

8 Pages of claims

4 Sheets of drawing

WARNING: ***DO NOT*** *submit original drawings. A high quality copy of the drawings should be supplied when filing a patent application. The drawings that are submitted to the Office must be on strong, white, smooth, and non-shiny paper and meet the standards according to § 1.84. If corrections to the drawings are necessary, they should be made to the original drawing and a high-quality copy of the corrected original drawing then submitted to the Office. Only one copy is required or desired. For comments on proposed then-new 37 C.F.R. § 1.84, see Notice of March 9, 1988 (1990 O.G. 57-62).*

NOTE: "Identifying indicia, if provided, should include the application number or the title of the invention, inventor's name, docket number (if any), and the name and telephone number of a person to call if the Office is unable to match the drawings to the proper application. This information should be placed on the back of each sheet of drawing a minimum distance of 1.5 cm. (5/8 inch) down from the top of the page . . ." 37 C.F.R. § 1.84(c)).

(complete the following, if applicable)

☐ The enclosed drawing(s) are photograph(s), and there is also attached a "PETITION TO ACCEPT PHOTOGRAPH(S) AS DRAWING(S)." 37 C.F.R. § 1.84(b).

☐ formal

☒ informal

B. Other Papers Enclosed

3 Pages of declaration and power of attorney

1 Pages of abstract

____ Other

4. Additional papers enclosed

☐ Amendment to claims

☐ Cancel in this applications claims _____ before calculating the filing fee. (At least one original independent claim must be retained for filing purposes.)

☐ Add the claims shown on the attached amendment. (Claims added have been numbered consecutively following the highest numbered original claims.)

☐ Preliminary Amendment

☐ Information Disclosure Statement (37 C.F.R. § 1.98)

☐ Form PTO–1449 (PTO/SB/08A and 08B)

☐ Citations

☐ Declaration of Biological Deposit

☒ Submission of "Sequence Listing," computer readable copy and/or amendment pertaining thereto for biotechnology invention containing nucleotide and/or amino acid sequence.

☐ Authorization of Attorney(s) to Accept and Follow Instructions from Representative

☐ Special Comments

☒ Other Statement of Submitting Sequence; Papercopy of Sequence

5. Declaration or oath (including power of attorney)

NOTE: A newly executed declaration is not required in a continuation or divisional application provided that the prior nonprovisional application contained a declaration as required, the application being filed is by all or fewer than all the inventors named in the prior application, there is no new matter in the application being filed, and a copy of the executed declaration filed in the prior application (showing the signature or an indication thereon that it was signed) is submitted. The copy must be accompanied by a statement requesting deletion of the names of person(s) who are not inventors of the application being filed. If the declaration in the prior application was filed under § 1.47, then a copy of that declaration must be filed accompanied by a copy of the decision granting § 1.47 status or, if a nonsigning person under § 1.47 has subsequently joined in a prior application, then a copy of the subsequently executed declaration must be filed. See 37 C.F.R. §§ 1.63(d)(1)–(3).

NOTE: A declaration filed to complete an application must be executed, identify the specification to which it is directed, identify each inventor by full name including family name and at least one given name, without abbreviation together with any other given name or initial, and the residence, post office address and country or citizenship of each inventor, and state whether the inventor is a sole or joint inventor. 37 C.F.R. § 1.63(a)(1)–(4).

☒ Enclosed

Executed by

(check **all** *applicable boxes)*

☒ inventor(s).

☐ legal representative of inventor(s). 37 C.F.R. §§ 1.42 or 1.43.

☐ joint inventor or person showing a proprietary interest on behalf of inventor who refused to sign or cannot be reached.

☐ This is the petition required by 37 C.F.R. § 1.47 and the statement required by 37 C.F.R. § 1.47 is also attached. *See* item 13 below for fee.

☐ Not Enclosed.

NOTE: Where the filing is a completion in the U.S. of an International Application or where the completion of the U.S. application contains subject matter in addition to the International Application, the application may be treated as a continuation or continuation-in-part, as the case may be, utilizing ADDED PAGE FOR NEW APPLICATION TRANSMITTAL WHERE BENEFIT OF PRIOR U.S. APPLICATION CLAIMED.

☐ Application is made by a person authorized under 37 C.F.R. § 1.41(c) on behalf of *all* the above named inventor(s).

(The declaration or oath, along with the surcharge required by 37 C.F.R. § 1.16(e) can be filed subsequently).

☐ Showing that the filing is authorized. *(not required unless called into question. 37 C.F.R. § 1.41(d))*

6. Inventorship Statement

WARNING: *If the named inventors are each not the inventors of all the claims an explanation, including the ownership of the various claims at the time the last claimed invention was made, should be submitted.*

The inventorship for all the claims in this application are:

☐ The same.

or

☐ Not the same. An explanation, including the ownership of the various claims at the time the last claimed invention was made,

☐ is submitted.

☐ will be submitted.

7. Language

NOTE: An application including a signed oath or declaration may be filed in a language other than English. An English translation of the non-English language application and the processing fee of $130.00 required by 37 C.F.R. § 1.17(k) is required to be filed with the application, or within such time as may be set by the Office. 37 C.F.R. § 1.52(d).

☒ English

☐ Non-English

☐ The attached translation includes a statement that the translation is accurate. 37 C.F.R. § 1.52(d).

8. Assignment

☒ An assignment of the invention to New England Biolabs, Inc.

☒ is attached. A separate ☐ "COVER SHEET FOR ASSIGNMENT (DOCUMENT) ACCOMPANYING NEW PATENT APPLICATION" or ☒ FORM PTO 1595 is also attached.

☐ will follow.

NOTE: "If an assignment is submitted with a new application, send two separate letters-one for the application and one for the assignment." Notice of May 4, 1990 (1114 O.G. 77-78).

WARNING: *A newly executed "CERTIFICATE UNDER 37 C.F.R. § 3.73(b)" must be filed when a continuation-in-part application is filed by an assignee. Notice of April 30, 1993, 1150 O.G. 62-64.*

9. Certified Copy

Certified copy(ies) of application(s)

Country	Appln. No.	Filed
Country	Appln. No.	Filed
Country	Appln. No.	Filed

from which priority is claimed

☐ is (are) attached.

☐ will follow.

NOTE: The foreign application forming the basis for the claim for priority must be referred to in the oath or declaration. 37 C.F.R. § 1.55(a) and 1.63.

NOTE: This item is for any foreign priority for which the application being filed directly relates. If any parent U.S. application or International Application from which this application claims benefit under 35 U.S.C. § 120 is itself entitled to priority from a prior foreign application, then complete item 18 on the ADDED PAGES FOR NEW APPLICATION TRANSMITTAL WHERE BENEFIT OF PRIOR U.S. APPLICATION(S) CLAIMED.

10. Fee Calculation (37 C.F.R. § 1.16)

A. ☒ Regular application

CLAIMS AS FILED						
Number filed		Number Extra		Rate	Basic Fee 37 C.F.R. 1.16(a) $760.00	
Total Claims (37 C.F.R. § 1.16(c))	49	− 20 =	29	×	$ 18.00	522.00
Independent Claims (37 C.F.R. § 1.16(b))	7	− 3 =	4	×	$ 78.00	312.00
Multiple dependent claim(s), if any (37 C.F.R. § 1.16(d))				+	$260.00	260.00

☐ Amendment cancelling extra claims is enclosed.

☐ Amendment deleting multiple-dependencies is enclosed.

☐ Fee for extra claims is not being paid at this time.

NOTE: If the fees for extra claims are not paid on filing they must be paid or the claims cancelled by amendment, prior to the expiration of the time period set for response by the Patent and Trademark Office in any notice of fee deficiency. 37 C.F.R. § 1.16(d).

Filing Fee Calculation $ 1854.00

B. ☐ Design application
($310.00—37 C.F.R. § 1.16(f))

Filing Fee Calculation $_____

C. ☐ Plant application
($480.00—37 C.F.R. § 1.16(g))

Filing fee calculation $_____

11. Small Entity Statement(s)

☒ Statement(s) that this is a filing by a small entity under 37 C.F.R. § 1.9 and 1.27 is (are) attached.

WARNING: *"Status as a small entity must be specifically established in each application or patent in which the status is available and desired. Status as a small entity in one application or patent does not affect any other application or patent, including applications or patents which are directly or indirectly dependent upon the application or patent in which the status has been established. The refiling of an application under § 1.53 as a continuation, division, or continuation-in-part (including a continued prosecution application under § 1.53(d)), or the filing of a reissue application requires a new determination as to continued entitlement to small entity status for the continuing or reissue application. A nonprovisional application claiming benefit under 35 U.S.C. § 119(e), 120, 121, or 365(c) of a prior application, or a reissue application may rely on a statement filed in the prior application or in the patent if the nonprovisional application or the reissue application includes a reference to the statement in the prior application or in the patent or includes a copy of the statement in the prior application or in the patent and status as a small entity is still proper and desired. The payment of the small entity basic statutory filing fee will be treated as such a reference for purposes of this section." 37 C.F.R. § 1.28(a)(2).*

WARNING: *"Small entity status must not be established when the person or persons signing the . . . statement can **unequivocally** make the required self-certification." M.P.E.P., § 509.03, 6th ed., rev. 2, July 1996 (emphasis added).*

(complete the following, if applicable)

☐ Status as a small entity was claimed in prior application

_____ / _____, filed on _____, from which benefit is being claimed for this application under:

35 U.S.C. § ☐ 119(e),
☐ 120,
☐ 121,
☐ 365(c),

and which status as a small entity is still proper and desired.

☐ A copy of the statement in the prior application is included.

Filing Fee Calculation (50% of **A, B** or **C** above)

$ 927.00

NOTE: Any excess of the full fee paid will be refunded if small entitiy status is established and a refund request are filed within 2 months of the date of timely payment of a full fee. The two-month period is not extendable under § 1.136. 37 C.F.R. § 1.28(a).

12. Request for International-Type Search (37 C.F.R. § 1.104(d))

(complete, if applicable)

☐ Please prepare an international-type search report for this application at the time when national examination on the merits takes place.

13. Fee Payment Being Made at This Time

☐ Not Enclosed

☐ No filing fee is to be paid at this time.
(This and the surcharge required by 37 C.F.R. § 1.16(e) can be paid subsequently.)

☒ Enclosed

☒ Filing fee	$ 927.00
☒ Recording assignment ($40.00; 37 C.F.R. § 1.21(h)) (See attached "COVER SHEET FOR ASSIGNMENT ACCOMPANYING NEW APPLICATION".)	$ 40.00
☐ Petition fee for filing by other than all the inventors or person on behalf of the inventor where inventor refused to sign or cannot be reached ($130.00; 37 C.F.R. §§ 1.47 and 1.17(i))	$ _____
☐ For processing an application with a specification in a non-English language ($130.00; 37 C.F.R. §§ 1.52(d) and 1.17(k))	$ _____
☐ Processing and retention fee ($130.00; 37 C.F.R. §§ 1.53(d) and 1.21(l))	$ _____
☐ Fee for international-type search report ($40.00; 37 C.F.R. § 1.21(e))	$ _____

NOTE: 37 C.F.R. § 1.21(l) establishes a fee for processing and retaining any application that is abandoned for failing to complete the application pursuant to 37 C.F.R. § 1.53(f) and this, as well as the changes to 37 C.F.R. §§ 1.53 and 1.78(a)(1), indicate that in order to obtain the benefit of a prior U.S. application, either the basic filing fee must be paid, or the processing and retention fee of § 1.21(l) must be paid, within 1 year from notification under § 53(f).

Total fees enclosed $ 967.00

14. Method of Payment of Fees

☒ Check in the amount of $ 967.00

☐ Charge Account No. _____ in the amount of $_____.

A duplicate of this transmittal is attached.

NOTE: Fees should be itemized in such a manner that it is clear for which purpose the fees are paid. 37 C.F.R. § 1.22(b).

15. Authorization to Charge Additional Fees

WARNING: *If no fees are to be paid on filing, the following items should not be completed.*

WARNING: *Accurately count claims, especially multiple dependent claims, to avoid unexpected high charges, if extra claim charges are authorized.*

☒ The Commissioner is hereby authorized to charge the following additional fees by this paper and during the entire pendency of this application to Account No. 14-0740 :

☒ 37 C.F.R. § 1.16(a), (f) or (g) (filing fees)

☒ 37 C.F.R. § 1.16(b), (c) and (d) (presentation of extra claims)

NOTE: Because additional fees for excess or multiple dependent claims not paid on filing or on later presentation must only be paid or these claims cancelled by amendment prior to the expiration of the time period set for response by the PTO in any notice of fee deficiency (37 C.F.R. § 1.16(d)), it might be best not to authorize the PTO to charge additional claim fees, except possibly when dealing with amendments after final action.

☒ 37 C.F.R. § 1.16(e) (surcharge for filing the basic filing fee and/or declaration on a date later than the filing date of the application)

☒ 37 C.F.R. § 1.17(a)(1)–(5) (extension fees pursuant to § 1.136(a)).

☒ 37 C.F.R. § 1.17 (application processing fees)

NOTE: ". . .A written request may be submitted in an application that is an authorization to treat any concurrent or future reply, requiring a petition for an extension of time under this paragraph for its timely submission, as incorporating a petition for extension of time for the appropriate length of time. An authorization to charge all required fees, fees under § 1.17, or all required extension of time fees will be treated as a constructive petition for an extension of time in any concurrent or future reply requiring a petition for an extension of time under this paragraph for its timely submission. Submission of the fee set forth in § 1.17(a) will also be treated as a constructive petition for an extension of time in any concurrent reply requiring a petition for an extension of time under this paragraph for its timely submission." 37 C.F.R. § 1.136(a)(3).

☐ 37 C.F.R. § 1.18 (issue fee at or before mailing of Notice of Allowance, pursuant to 37 C.F.R. § 1.311(b))

NOTE: Where an authorization to charge the issue fee to a deposit account has been filed before the mailing of a Notice of Allowance, the issue fee will be automatically charged to the deposit account at the time of mailing the notice of allowance. 37 C.F.R. § 1.311(b).

NOTE: 37 C.F.R. § 1.28(b) requires "Notification of any change in status resulting in loss of entitlement to small entity status must be filed in the application . . . prior to paying, or at the time of paying, . . . the issue fee. . . " From the wording of 37 C.F.R. § 1.28(b), (a) notification of change of status must be made even if the fee is paid as "other than a small entity" and (b) no notification is required if the change is to another small entity.

16. Instructions as to Overpayment

NOTE: ". . . Amounts of twenty-five dollars or less will not be returned unless specifically requested within a reasonable time, nor will the payer be notified of such amounts; amounts over twenty-five dollars may be returned by check or, if requested, by credit to a deposit account." 37 C.F.R. § 1.26(a).

☒ Credit Account No. 14-0740

☐ Refund

SIGNATURE OF PRACTITIONER

Reg. No. 30901

Gregory D. Williams
General Counsel

(type or print name of attorney)

Tel. No. (978) 927-5054 X:292

New England Biolabs, Inc.
32 Tozer Road

P.O. Address

Customer No.

Beverly, MA 01915

☒ **Incorporation by reference of added pages**

(check the following item if the application in this transmittal claims the benefit of prior U.S. application(s) (including an international application entering the U.S. stage as a continuation, divisional or C-I-P application) and complete and attach the ADDED PAGES FOR NEW APPLICATION TRANSMITTAL WHERE BENEFIT OF PRIOR U.S. APPLICATION(S) CLAIMED)

☒ Plus Added Pages for New Application Transmittal Where Benefit of Prior U.S. Application(s) Claimed

Number of pages added 5

☐ Plus Added Pages for Papers Referred to in Item 4 Above

Number of pages added _____

☐ Plus added pages deleting names of inventor(s) named in prior application(s) who is/are no longer inventor(s) of the subject matter claimed in this application.

Number of pages added _____

☐ Plus "Assignment Cover Letter Accompanying New Application"

Number of pages added _____

☐ **Statement Where No Further Pages Added**

(if no further pages form a part of this Transmittal, then end this Transmittal with this page and check the following item)

☐ This transmittal ends with this page.

Practitioner's Docket No. NEB-154 **PATENT**

ADDED PAGES FOR APPLICATION TRANSMITTAL WHERE BENEFIT OF PRIOR U.S. APPLICATION(S) CLAIMED

NOTE: See 37 C.F.R. § 1.78.

17. Relate Back

WARNING: *If an application claims the benefit of the filing date of an earlier filed application under 35 U.S.C. §§ 120, 121 or 365(c), the 20-year term of that application will be based upon the filing date of the earliest U.S. application that the application makes reference to under 35 U.S.C. §§ 120, 121 or 365(c). (35 U.S.C. § 154(a)(2) does not take into account, for the determination of the patent term, any application on which priority is claimed under 35 U.S.C. §§ 119, 365(a) or 365(b).) For a c-i-p application, applicant should review whether any claim in the patent that will issue is supported by an earlier application and, if not, the applicant should consider canceling the reference to the earlier filed application. The term of a patent is not based on a claim-by-claim approach. See Notice of April 14, 1995, 60 Fed. Reg. 20,195, at 20,205.*

(complete the following, if applicable)

☐ Amend the specification by inserting, before the first line, the following sentence:

A. 35 U.S.C. § 119(e)

NOTE: "Any nonprovisional application claiming the benefit of one or more prior filed copending provisional applications must contain or be amended to contain in the first sentence of the specification following the title a reference to each such prior provisional application, identifying it as a provisional application, and including the provisional application number (consisting of series code and serial number)." 37 C.F.R. § 1.78(a)(4).

☒ "This application claims the benefit of U.S. Provisional Application(s) No(s).:

APPLICATION NO(S).:	FILING DATE
60 / 102,413	Sept. 30, 1998 "
/	"
/	"

B. 35 U.S.C. §§ 120, 121 and 365(c)

NOTE: "Except for a continued prosecution application filed under § 1.53(d), any nonprovisional application claiming the benefit of one or more prior filed copending nonprovisional applications or international applications designating the United States of America must contain or be amended to contain in the first sentence of the specification following the title a reference to each such prior application, identifying it by application number (consisting of the series code and serial number) or international application number and international filing date and indicating the relationship of the applications. . . . Cross-references to other related applications may be made when appropriate." (See § 1.14(a)). 37 C.F.R. § 1.78(a)(2).

☒ "This application is a

- ☐ continuation
- ☒ continuation-in-part
- ☐ divisional

of copending application(s)

☒ application number 08/ 811,492 (now U.S. Patent No. 5,834,247 issued November 10, 1998) ~~filed on~~ filed on March 5, 1997."

☐ International Application _____ filed on _____ and which designated the U.S."

NOTE: The proper reference to a prior filed PCT application that entered the U.S. national phase is the U.S. serial number and the filing date of the PCT application that designated the U.S.

NOTE: (1) Where the application being transmitted adds subject matter to the International Application, then the filing can be as a continuation-in-part or (2) if it is desired to do so for other reasons then the filing can be as a continuation.

NOTE: The deadline for entering the national phase in the U.S. for an international application was clarified in the Notice of April 28, 1987 (1079 O.G. 32 to 46) as follows:

"The Patent and Trademark Office considers the International application to be pending until the 22nd month from the priority date if the United States has been designated and no Demand for International Preliminary Examination has been filed prior to the expiration of the 19th month from the priority date and until the 32nd month from the priority date if a Demand for International Preliminary Examination which elected the United States of America has been filed prior to the expiration of the 19th month from the priority date, provided that a copy of the international application has been communicated to the Patent and Trademark Office within the 20 or 30 month period respectively. If a copy of the international application has not been communicated to the Patent and Trademark Office within the 20 or 30 month period respectively, the international application becomes abandoned as to the United States 20 or 30 months from the priority date respectivley. These periods have been placed in the rules as paragraph (h) of § 1.494 and paragraph (i) of § 1.495. A continuing application under 35 U.S.C. 365(c) and 120 may be filed anytime during the pendency of the international application."

☐ "The nonprovisional application designated above, namely application _____ / _____, filed _____, claims the benefit of U.S. Provisional Application(s) No(s).:

APPLICATION NO(S).:	**FILING DATE**
_____ /_____	_____ "
_____ /_____	_____ "
_____ /_____	_____ "

☐ Where more than one reference is made above, please combine all references into one sentence.

18. Relate Back—35 U.S.C. § 119 Priority Claim for Prior Application

The prior U.S. application(s), including any prior International Application designating the U.S., identified above in item 17B, in turn itself claim(s) foreign priority(ies) as follows:

Country	Appln. no.	Filed on

The certified copy(ies) has (have)

☐ been filed on _____, in prior application 0 /_____, which was filed on _____.

☐ is (are) attached.

WARNING: *The certified copy of the priority application that may have been communicated to the PTO by the International Bureau may* ***not*** *be relied on without any need to file a certified copy of the priority application* ***in the continuing application.*** *This is so because the certified copy of the priority application communicated by the International Bureau is placed in a folder and is not assigned a U.S. serial number unless the national stage is entered. Such folders are disposed of if the national stage is not entered. Therefore, such certified copies may not be available if needed later in the prosecution of a continuing application. An alternative would be to physically remove the priority documents from the folders and transfer them to the continuing application. The resources required to request transfer, retrieve the folders, make suitable record notations, transfer the certified copies, enter and make a record of such copies in the Continuing Application are substantial. Accordingly, the priority documents in folders of international applications that have not entered the national stage may not be relied on. Notice of April 28, 1987 (1079 O.G. 32 to 46).*

19. Maintenance of Copendency of Prior Application

NOTE: The PTO finds it useful if a copy of the petition filed in the prior application extending the term for response is filed with the papers constituting the filing of the continuation application. Notice of November 5, 1985 (1060 O.G. 27).

A. ☐ Extension of time in prior application

(This item ***must*** *be completed and the papers filed* ***in the prior application,*** *if the period set in the prior application has run.)*

☐ A petition, fee and response extends the term in the pending **prior** application until _____.

☐ A **copy** of the petition filed in prior application is attached.

B. ☐ Conditional Petition for Extension of Time in Prior Application

(complete this item, if previous item not applicable)

☐ A conditional petition for extension of time is being filed in the pending **prior** application.

☐ A **copy** of the conditional petition filed in the prior application is attached.

20. Further Inventorship Statement Where Benefit of Prior Application(s) Claimed

(complete applicable item (a), (b) and/or (c) below)

(a) ☐ This application discloses and claims only subject matter disclosed in the prior application whose particulars are set out above and the inventor(s) in this application are

- ☐ the same.
- ☐ less than those named in the prior application. It is requested that the following inventor(s) identified for the prior application be deleted:

_____

(type name(s) of inventor(s) to be deleted)

(b) ☐ This application discloses and claims additional disclosure by amendment and a new declaration or oath is being filed. With respect to the prior application, the inventor(s) in this application are

- ☐ the same.
- ☐ the following additional inventor(s) have been added:

_____

(type name(s) of inventor(s) to be added)

(c) The inventorship for all the claims in this application are

- ☐ the same.
- ☐ not the same. An explanation, including the ownership of the various claims at the time the last claimed invention was made
  - ☐ is submitted.
  - ☐ will be submitted.

21. Abandonment of Prior Application *(if applicable)*

☐ Please abandon the prior application at a time while the prior application is pending, or when the petition for extension of time or to revive in that application is granted, and when this application is granted a filing date, so as to make this application copending with said prior application.

NOTE: According to the Notice of May 13, 1983 (103, TMOG 6-7), the filing of a continuation or continuation-in-part application is a proper response with respect to a petition for extension of time or a petition to revive and should include the express abandonment of the prior application conditioned upon the granting of the petition and the granting of a filing date to the continuing application.

22. Petition for Suspension of Prosecution for the Time Necessary to File an Amendment

WARNING: *"The claims of a new application may be finally rejected in the first Office action in those situations where (1) the new application is a continuing application of, or a substitute for, an earlier application, and (2) all the claims of the new application (a) are drawn to the same invention claimed in the earlier application, and (b) would have been properly finally rejected on the grounds of art of record in the next Office action if they had been entered in the earlier application." M.P.E.P., § 706.07(b), 6th ed., rev. 2.*

NOTE: Where it is possible that the claims on file will give rise to a first action final for this continuation application and for some reason an amendment cannot be filed promptly (e.g., experimental data is being gathered) it may be desirable to file a petition for suspension of prosecution for the time necessary.

(check the next item, if applicable)

☐ There is provided herewith a Petition To Suspend Prosecution for the Time Necessary to File An Amendment (New Application Filed Concurrently)

23. Small Entity (37 C.F.R. § 1.28(a))

☐ Applicant has established small entity status by the filing of a statement in parent application /_____ on _____ .

☐ A copy of the statement previously filed is included.

WARNING: *See 37 C.F.R. § 1.28(a).*

WARNING: *"Small entity status must not be established when the person or persons signing the . . . statement can **unequivocally** make the required self-certification." M.P.E.P., § 509.03, 6th ed., rev. 2, July 1996 (emphasis added).*

24. NOTIFICATION IN PARENT APPLICATION OF THIS FILING

☐ A notification of the filing of this
(check one of the following)

☐ continuation

☐ continuation-in-part

☐ divisional

is being filed in the parent application, from which this application claims priority under 35 U.S.C. § 120.

Practitioner's Docket No. NEB-154 **PATENT**

☒ Applicant Evans, et al. ☐ Patentee _____

☐ Application No. ☐ Patent No. _____

☐ Filed on ☐ Issued on _____

Title: INTEIN-MEDIATED PROTEIN LIGATION OF EXPRESSED PROTEINS

STATEMENT CLAIMING SMALL ENTITY STATUS (37 CFR 1.9(f) and 1.27(c))—SMALL BUSINESS CONCERN

I hereby state that I am

☐ the owner of the small business concern identified below:

☒ an official of the small business concern empowered to act on behalf of the concern identified below:

Name of Small Business Concern New England Biolabs, Inc.

Address of Small Business Concern 32 Tozer Road

Beverly, MA 01915

I hereby state that the above identified small business concern qualifies as a small business concern, as defined in 13 CFR 121.12, and reproduced in 37 CFR 1.9(d), for purposes of paying reduced fees to the United States Patent and Trademark Office under Sections 41(a) and (b) of Title 35, United States Code, in that the number of employees of the concern, including those of its affiliates, does not exceed 500 persons. For purposes of this statement, (1) the number of employees of the business concern is the average over the previous fiscal year of the concern of the persons employed on a full-time, part-time or temporary basis during each of the pay periods of the fiscal year, and (2) concerns are affiliates of each other when either, directly or indirectly, one concern controls or has the power to control the other, or a third-party or parties controls or has the power to control both.

I hereby state that rights under contract or law have been conveyed to, and remain with, the small business concern identified above, with regard to the invention described in

☒ the specification filed herewith, with title as listed above.

☐ the application identified above.

☐ the patent identified above.

If the rights held by the above-identified small business concern are not exclusive, each individual, concern or organization having rights in the invention is listed below* and no rights to the invention are held by any person, other than the inventor, who would not qualify as an independent inventor under 37 CFR 1.9(c), if that person made the invention, or by any concern which would not qualify as a small business concern under 37 CFR 1.9(d) or a nonprofit organization under 37 CFR 1.9(e).

*NOTE: *Separate statements are required from each named person, concern or organization having rights to the invention as to their status as small entities. (37 CFR 1.27)*

Each such person, concern or organization having any rights in the invention is listed below:

☐ No such person, concern, or organization exists.

☐ Each such person, concern or organization is listed below.

Name New England Biolabs, Inc.

Address 32 Tozer Road

Beverly, MA 01915

☐ INDIVIDUAL ☒ SMALL BUSINESS CONCERN ☐ NONPROFIT ORGANIZATION

Name _____

Address _____

☐ INDIVIDUAL ☐ SMALL BUSINESS CONCERN ☐ NONPROFIT ORGANIZATION

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small business entity is no longer appropriate. (37 CFR 1.28(b))

(check the following item, if desired)

NOTE: The following verification statement need not be made in accordance with the rules published on Oct. 10, 1997, 62 Fed. Reg. 52,131, effective Dec. 1, 1997.

NOTE: "The presentation to the Office (whether by signing, filing, submitting, or later advocating) of any paper by a party, whether a practitioner or non-practitioner, constitutes a certification under § 10.18(b) of this chapter. Violations of § 10.18(b)(2) of this chapter by a party, whether a practitioner or non-practitioner, may result in the imposition of sanctions under § 10.18(c) of this chapter. Any practitioner violating § 10.18(b) may also be subject to disciplinary action. See §§ 10.18(d) and 10.23(c)(15)." 37 C.F.R. § 1.4(d)(2).

☒ I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further, that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

Name of Person Signing Gregory D. Williams

Title of Person if Other Than Owner General Counsel

Address of Person Signing New England Biolabs, Inc.
32 Tozer Road; Beverly, MA 01915

SIGNATURE _____ Date 2/12/99

Docket No.: NEB-154

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE
APPLICATION FOR UNITED STATES LETTES PATENT

INVENTORS: Thomas C. Evans, Jr.
Ming-Qun Xu

TITLE: INTEIN-MEDIATED PROTEIN LIGATION OF EXPRESSED PROTEINS

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INTEIN-MEDIATED PROTEIN LIGATION OF EXPRESSED PROTEINS

RELATED APPLICATIONS

This Application is a Continuation-In-Part of U.S.S.N. 08/811,492, filed March 5, 1997 now U.S. Patent No. 5,834,247, issued November 10, 1998, entitled "Modified Proteins Comprising Controllable Intervening Protein Sequences Or Their Elements Methods of Producing Same and Methods For Purification Of A Target Protein Comprised By A Modified Protein", and of U.S.S.N. 60/102,413, filed September 30, 1998, entitled "Intein Mediated Peptide Ligation."

BACKGROUND OF THE INVENTION

The present invention relates to methods of intein-mediated ligation of proteins. More specifically, the present invention relates to intein-mediated ligation of expressed proteins containing a predetermined N-terminal residue and/or a C-terminal thioester generated via use of one or more naturally occurring or modified inteins. Preferably, the predetermined residue is cysteine.

Inteins are the protein equivalent of the self-splicing RNA introns (see Perler et al., *Nucleic Acids Res.* 22:1125-1127 (1994)), which catalyze their own excision from a precursor protein with the concomitant fusion of the flanking protein sequences, known as exteins (reviewed in Perler et al.,

Curr. Opin. Chem. Biol. 1:292-299 (1997); Perler, F. B. *Cell* 92(1):1-4 (1998); Xu et al.,*EMBO J.* 15(19):5146-5153 (1996)).

Studies into the mechanism of intein splicing led to the development of a protein purification system that utilized thiol-induced cleavage of the peptide bond at the N-terminus of the *Sce* VMA intein (Chong et al., *Gene* 192(2):271-281 (1997)). Purification with this intein-mediated system generates a bacterially-expressed protein with a C-terminal thioester (Chong et al., (1997)). In one application, where it is described to isolate a cytotoxic protein, the bacterially expressed protein with the C-terminal thioester is then fused to a chemically-synthesized peptide with an N-terminal cysteine using the chemistry described for "native chemical ligation" (Evans et al., *Protein Sci.* 7:2256-2264 (1998); Muir et al., *Proc. Natl. Acad. Sci. USA* 95:6705-6710 (1998)).

This technique, referred to as "intein-mediated protein ligation" (IPL), represents an important advance in protein semi-synthetic techniques. However, because chemically-synthesized peptides of larger than about 100 residues are difficult to obtain, the general application of IPL is limited by the requirement of a chemically-synthesized peptide as a ligation partner.

IPL technology would be significantly expanded if an expressed protein with a predetermined N-terminus, such as cysteine, could be generated. This would allow the fusion of

one or more expressed proteins from a host cell, such as bacterial, yeast or mammalian cells.

One method of generating an N-terminal cysteine is with the use of proteases. However, proteases have many disadvantages, such as the possibility of multiple protease sites within a protein, as well as the chance of non-specific degradation. Furthermore, following proteolysis, the proteases must be inactivated or purified away from the protein of interest before proceeding with IPL. (Xu, et al., *Proc. Natl. Acad. Sci. USA* 96(2):388-393 (1999) and Erlandson, et al., *Chem. Biol.*, 3:981-991 (1996))

There is, therefore, a need for an improved intein-mediated protein ligation method which overcomes the noted limitations of current IPL methods and which eliminates the need for use of proteases to generate an N-terminal cysteine residue. Such an improved IPL method would have widespread applicability for the ligation of expressed proteins, for example, labeling of extensive portions of a protein for, among other things, NMR analysis.

SUMMARY OF THE INVENTION

In accordance with the present invention, there is provided a method for the ligation of expressed proteins utilizing one or more inteins which display cleavage at their N- and/or C-termini. In accordance with the present

invention, such inteins may occur either naturally or may be modified to cleave at their N- and/or C-termini. Inteins displaying N- and/or C-terminal cleavage enable the facile isolation of a protein having a C-terminal thioester and a protein having an N-terminal amino acid residue such as cysteine, respectively, for use in the fusion of one or more expressed proteins. Alternatively, the method may be used to generate a single protein having both a C-terminal thioester and a specified N-terminal amino acid residue, such as cysteine, for the creation of cyclic or polymerized proteins. These methods involve the steps of generating at least one C-terminal thioester-tagged first target protein, generating at least one second target protein having a specified N-terminal amino acid residue, for example cysteine, and ligating these proteins. This method may be used where a single protein is expressed, where, for example, the C-terminal thioester end of the protein is fused to the N-terminal end of the same protein. The method may further include chitin-resin purification steps.

In one preferred embodiment the intein from the RIR1 *Methanobacterium thermoautotrophicum* is modified to cleave at either the C-terminus or N-terminus. The modified intein allows for the release of a bacterially expressed protein during a one-column purification, thus eliminating the need proteases entirely. DNA encoding these modified inteins and plasmids containing these modified inteins are also provided by the instant invention.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a diagram depicting both the N-terminal and C-terminal cleavage reactions which comprise intein-mediated protein ligation. The modified *Mth* RIR1 intein was used to purify both MBP with a C-terminal thioester and T4 DNA ligase with an N-terminal cysteine. The *Mth* RIR1 intein for N-terminal cleavage, intein(N), carried the $P^{-1}G/N^{134}A$ double mutation. The full length fusion protein consisting of MBP-intein(N)-CBD was separated from cell extract by binding the CBD portion of the fusion protein to a chitin resin. Overnight incubation in the presence of 100 mM 2-mercaptoethanesulfonic acid (MESNA) induced cleavage of the peptide bond prior to the N-terminus of the intein and created a thioester on the C-terminus of MBP. The C-terminal cleavage vector, intein(C), had the $P^{-1}G/C^{1}A$ double mutation. The precursor CBD-intein(C)-T4 DNA ligase was isolated from induced *E. coli* cell extract by binding to a chitin resin as described for N-terminal cleavage. Fission of the peptide bond following the C-terminal residue of the intein at a preferred temperature and pH resulted in the production of T4 DNA ligase with an N-terminal cysteine. Ligation occurred when the proteins containing the complementary reactive groups were mixed and concentrated, resulting in a native peptide bond between the two reacting species.

Figure 2A is a gel depicting the purification of a C-terminal thioester-tagged maltose binding protein (MBP) *via* a thiol-inducible *Mth* RIR1 intein construct pMRB10G (containing the modified intein, R(N), with $P^{-1}G/N^{134}A$ mutation) and the purification of T4 DNA ligase having an N-terminal cysteine using the vector pBRL-A (containing the modified intein, R(C), with $P^{-1}G/C^{1}A$ mutation). Lanes 1-3, purification of maltose binding protein (MBP) (M, 43 kDa) with a C-terminal thioester. Lane 1. ER2566 cells transformed with plasmid pMRB10G following Isopropyl ß-D-thiogalactopyranoside (IPTG) induction. Lane 2. Cell extract after passage over a chitin resin. Note that the fusion protein, M-R(N)-B, binds to the resin, where B is the chitin binding domain. Lane 3. Fraction 3 of the elution from the chitin resin following overnight incubation at 4°C in the presence of 100 mM MESNA. Lanes 4-6, purification of T4 DNA ligase (L, 56 kDa) with an N-terminal cysteine. Lane 4. IPTG induced ER2566 cells containing plasmid pBRL-A. Lane 5. Cell extract after application to a chitin resin. B-R(C)-L, the fusion protein, binds to the resin. Lane 6. Elution of T4 DNA ligase with an N-terminal cysteine after overnight incubation at room temperature in pH 7 buffer

Figure 2B is a gel depicting ligation of T4 DNA ligase having an N-terminal cysteine to a C-terminal thioester tagged MBP. Lane 1. Thioester-tagged MBP. Lane 2. T4 DNA ligase with an N-terminal cysteine. Lane 3. Ligation reaction

of MBP (0.8 mM) with T4 DNA ligase (0.8 mM), generating M-L, after overnight incubation at 4°C.

Figure 3 is a gel depicting the effect of induction temperature on the cleaving and/or splicing activity of the *Mth* RIR1 intein or *Mth* RIR1 intein mutants. The *Mth* RIR1 intein or mutants thereof, with 5 native N- and C-terminal extein residues were induced at either 15°C or 37°C. The intein was expressed as a fusion protein (M-R-B, 63 kDa) consisting of N-terminal maltose binding protein (M, 43 kDa), the *Mth* RIR1 intein (R, 15 kDa) and at its C-terminus was the chitin binding domain (B, 5 kDa). Lanes 1 and 2. M-R-B with the unmodified *Mth* RIR1 intein. Note the small amount of spliced product (M-B, 48 kDa). Lanes 3 and 4. *Mth* intein with Pro^{-1} replaced with Ala, M-R-B($P^{-1}A$). Both spliced product (M-B) and N-terminal cleavage product (M) are visible. Lanes 5 and 6. Replacement of Pro^{-1} with Gly (M-R-B($P^{-1}G$)) showed some splicing as well as N- and C-terminal cleavage, M and M-R, respectively. Lanes 7 and 8. The Pro^{-1} to Gly and Cys^{1} to Ser double mutant, M-R-B($P^{-1}G/C^{1}S$), displayed induction temperature dependent C-terminal cleavage (M-R) activity. Lanes 9 and 10. The M-R-B($P^{-1}G/N^{134}A$) mutant possessed only N-terminal cleavage activity producing M. The *Mth* intein or *Mth* intein -CBD fusion is not visible in this Figure.

Figure 4 is a nucleotide sequence (SEQ ID NO:23) comparison of wild type *Mth* RIR1 intein and synthetic *Mth*

RIR1 intein indicating the location of 61 silent base mutations designed to increase expression in *E. coli*. DNA alignment of the wild type *Mth* RIR1 intein (top strand) and the synthetic *Mth* RIR1 intein (bottom strand). To increase expression levels in *E. coli*, 61 silent base changes were made in 49 seperate codons when creating the synthetic gene. The first and last codons of the wild type *Mth* RIR1 intein are shown in bold.

DETAILED DESCRIPTION

The present invention provides a solution to the limitations of current intein-mediated ligation methods by eliminating the need for a synthetic peptide as a ligation partner, and providing a method which is suitable for the fusion one or more expressed proteins.

In general, any intein displaying N- and/or C-terminal cleavage at its splice junctions can be used to generate a defined N-terminus, such as cysteine as well as a C-terminal thioester for use in the fusion of expressed proteins. Inteins which may be used in practicing the present invention include those described in Perler, et al., *Nucleic Acids Res.*, 27(1):346-347 (1999).

In accordance with one preferred embodiment, an intein found in the ribonucleoside diphosphate reductase gene of *Methanobacterium thermoautotrophicum* (the *Mth* RIR1 intein)

was modified for the facile isolation of a protein with an N-terminal cysteine for use in the *in vitro* fusion of two bacterially-expressed proteins. The 134-amino acid *Mth* RIR1 intein is the smallest of the known mini-inteins, and may be close to the minimum amino acid sequence needed to promote splicing (Smith et.al., *J. Bacteriol.* 179: 7135-7155 (1997)).

The *Mth* RIR1 intein has a proline residue on the N-terminal side of the first amino acid of the intein. This residue was previously shown to inhibit splicing in the Sce VMA intein (Chong et al., *J. Biol. Chem.* 273:10567-10577 (1998)). The intein was found to splice poorly in *E. coli* when this naturally occurring proline is present. Splicing proficiency increases when this proline is replaced with an alanine residue. Constructs that display efficient N- and C-terminal cleavage are created by replacing either the C-terminal asparagine or N-terminal cysteine of the intein, respectively, with alanine.

These constructs allow for the formation of an intein-generated C-terminal thioester on a first target protein and an intein-generated N-terminal cysteine on a second target protein. These complementary reactive groups may then be ligated via native chemical ligation to produce a peptide bond (Evans et al *supra* (1998), Muir et al *supra* (1998)). Alternatively, a single protein containing both reactive groups may be generated for the creation of cyclic or polymerized

proteins. Likewise, more than one first or second target proteins may be generated via use of multiple mutant inteins.

As used herein, the terms fusion and ligation are used interchangeably. Also as used herein, protein shall mean any protein, fragment of any protein, or peptide capable of ligation according to the methods of the instant invention. Further, as used herein, target protein shall mean any protein the ligation of which, according to the methods of the instant invention, is desired.

The general method of intein-mediated protein ligation in accordance with the present invention is as follows:

(1) An intein of interest is isolated and cloned into an appropriate expression vector(s) such as bacterial, plant, insect, yeast and mammalian cells.

(2) The intein is engineered for N- and/or C-terminal cleavage unless the wild type intein displays the desired cleavage activities. In a preferred embodiment, a modified intein with the desired cleavage properties can be generated by substituting one or more residues within and/or flanking the intein sequence. For example, a modified intein having N-terminal cleavage activity can be created by changing the last intein residue. Alternatively, a modified intein with C-terminal cleavage activity can be created by changing the first intein residue.

(3) The intein with N- and/or C-terminal cleavage activity is fused with an affinity tag to allow purification away from other endogenous proteins.

(4) The intein or inteins, either wild type or modified, that display N-terminal and/or C-terminal cleavage, or both, are fused to the desired target protein coding region or regions upstream and/or downstream of the intein.

(5) An intein that cleaves at its N-terminus in a thiol reagent dependent manner is used to isolate a protein with a C-terminal thioester. This cleavage and isolation is, for example, carried out as previously described for the *Sce* VMA and *Mxe* GyrA inteins (Chong et al., *Gene* 192(2):271-281 (1997); Evans et al., *Protein Sci.* 7:2256-2264 (1998)). As discussed previously, multiple C-terminal thioester-tagged proteins may be generated at this step .

(6) A target protein having a specified N-terminus is generated by cleavage of a construct containing an intein that cleaves at its C-terminus. The specified N-terminal residue may be any of the amino acids, but preferably cysteine. As discussed previously, this step may alternately generate a specified N-terminal on the same protein containing a C-terminal thioester, to yield a single protein containing both reactive groups. Alternatively, multiple proteins having the specified N-terminus may be generated at this step.

(7) Thioester-tagged target protein and target protein having a specified N-termini are fused via intein-mediated protein ligation (IPL) (see Figure 2B). In a preferred embodiment, the N-terminus is cysteine. Alternatively, a single protein containing both a C-terminal thioester and a specified N-terminus, such as a cysteine, may undergo intramolecular ligation to yield a cyclic product and/or intermolecular ligation to yield polymerized proteins.

The methodology described by the instant invention significantly expands the utility of current IPL methods to enable the labeling of extensive portions of a protein for NMR analysis and the isolation of a greater variety of cytotoxic proteins. In addition, this advance opens the possibility of labeling the central portion of a protein by ligating three or more fragments.

The use of an intein or inteins with N-terminal and C-terminal cleavage activity provides the potential to create a defined N-terminus, such as a cysteine, and a C-terminal thioester on a single protein. The intramolecular ligation of the resulting protein generates a circular protein, whereas the intermolecular ligation of several of these proteins generates a protein polymer.

Cleavage at the N- and/or the C-terminus of an intein can be brought about by introducing changes to the intein

and/or its extein sequences. Also, naturally occuring inteins may display these properties and require no manipulation. Cleavage at the N- and/or C-terminus of an intein can occur uncontrollably or induced using nucleophilc compounds, such as thiol reagents, temperature, pH, salt, chaotropic agents, or any combination of the aforementioned conditions and/or reagents.

The Examples presented below are only intended as specific preferred embodiments of the present invention and are not intended to limit the scope of the invention except as provided in the claims herein. The present invention encompasses modifications and variations of the methods taught herein which would be obvious to one of ordinary skill in the art.

The references cited above and below are herein incorporated by reference.

EXAMPLE I

Creation of the *Mth* RIR1 synthetic gene

The gene encoding the *Mth* RIR1 intein along with 5 native N- and C-extein residues (Smith et al. *supra* (1997)) was constructed using 10 oligonucleotides (New England Biolabs, Beverly, MA) comprising both strands of the gene, as follows:

1) 5'-TCGAGGCAACCAACCCCTGCGTATCCGGTGACACCATTGT AATGACTAGTGGCGGTCCGCGCACTGTGGCTGAACTGGAG GGCAAACCGTTCACCGCAC-3' (SEQ ID NO:1)

2) 5'-CCGGTTGGCTGCTCGCCACAGTTGTGTACAATGAAGCCAT TAGCAGTGAATGCGCTAGCACCGTAAACAGTAGCGTCATA AACATCCTGGCGG-3' (SEQ ID NO:2)

3) 5'-pTGATTCGCGGCTCTGGCTACCCATGCCCCTCAGGTTTCTT CCGCACCTGTGAACGTGACGTATATGATCTGCGTACACGT GAGGGTCATTGCTTACGTTT-3' (SRQ ID NO:3)

4) 5'-pGACCCATGATCACCGTGTTCTGGTGATGGATGGTGGCCTG GAATGGCGTGCCGCGGGTGAACTGGAACGCGGCGACCGCC TGGTGATGGATGATGCAGCT-3' (SEQ ID NO:4)

5) 5'-pGGCGAGTTTCCGGCACTGGCAACCTTCCGTGGCCTGCGTG GCGCTGGCCGCCAGGATGTTTATGACGCTACTGTTTACGG TGCTAGC-3' (SEQ ID NO:5)

6) 5'-pGCATTCACTGCTAATGGCTTCATTGTACACAACTGTGGCG AGCAGCCAA-3' (SEQ ID NO:6)

7) 5'-pCCAGCGCCACGCAGGCCACGGAAGGTTGCCAGTGCCGGAA ACTCGCCAGCTGCATCATCCATCACCAGGCGGTCGCCGCG TTCCAGTTCACCCGCGGCAC-3' (SEQ ID NO:7)

8) 5'-pGCCATTCCAGGCCACCATCCATCACCAGAACACGGTGATC ATGGGTCAAACGTAAGCAATGACCCTCACGTGTACGCAGA TCATATACGT-3' (SEQ ID NO:8)

9) 5'-pCACGTTCACAGGTGCGGAAGAAACCTGAGGGGCATGGGTA GCCAGAGCCGCGAATCAGTGCGGTGAACGGTTTGCCCTCC AGTTCAGCCACAGTGCG-3' (SEQ ID NO:9)

10) 5'-pCGGACCGCCACTAGTCATTACAATGGTGTCACCGGATACG CAGGGGTTGGTTGCC-3' (SEQ ID NO:10)

To ensure maximal *E. coli* expression, the coding region of the synthetic *Mth* RIR1 intein incorporates 61 silent base mutations in 49 of the 134 codons (see Figure 4) in the wildtype *Mth* RIR1 intein gene (GenBank AE000845). The oligonucleotides were annealed by mixing at equimolar ratios (400 nM) in a ligation buffer (50 mM Tris-HCl, pH 7.5 containing 10 mM $MgCl_2$, 10 mM dithiothreitol, 1 mM ATP, and 25 μg BSA) followed by heating to 95°C. After cooling to room temperature, the annealed and ligated oligonucleotides were inserted into the *Xho*I and *Age*I sites of pMYB5 (NEB), replacing the *Sce* VMA intein and creating the plasmid pMRB8P.

Engineering the *Mth* RIR1 intein for N- and C-terminal cleavage

The unique *Xho*I and *Spe*I sites flanking the N-terminal splice junction and the unique *BsrG*I and *Age*I sites flanking the C-terminal splice junction allowed substitution of amino acid residues by linker replacement. The proline residue, Pro^{-1}, preceding the intein in pMRB8P was substituted with alanine or glycine to yield pMRB8A and pMRB8G1, respectively. Substitution of Pro^{-1}-Cys^{1} with Gly-Ser or Gly-Ala yielded pMRB9GS and pMRB9GA, respectively. Replacing Asn^{134} with Ala in pMRB8G1 resulted in pMRB10G. The following linkers were used for substitution of the native amino acids at the

splice junctions (each linker was formed by annealing two synthetic oligonucleotides as described above):

$P^{-1}A$ linker:	5'-TCGAGGCAACCAACGCATGCGTATCCGGT GACACCATTGTAATGA-3' (SEQ ID NO:11)
and	5'-CTAGTCATTACAATGGTGTCACCGGATAC GCATGCGTTGGTTGCC-3' (SEQ ID NO:12)
$P^{-1}G$ linker:	5'-TCGAGGGCTGCGTATCCGGTGACACCATT GTAATGA–3 (SEQ ID NO:13)'
and	5'-CTAGTCATTACAATGGTGTCACCGGATAC GCAGCCC-3' (SEQ ID NO:14)
$P^{-1}G/C^{1}S$ linker:	5'-TCGAGGGCATCGAGGCAACCAACGGATC CGTATCCGGTGACACCATTGTAATGA-3' (SEQ ID NO:15)
and	5'-CTAGTCATTACAATGGTGTCACCGGATAC GGATCCGTTGGTTGCCTCGATGCCC-3' (SEQ ID NO:16)
$P^{-1}G/C^{1}A$ linker:	5'-TCGAGGGCATCGAGGCAACCAACGGCGCC GTATCCGGTGACACCATTGTAATGA -3' (SEQ ID NO:17)
and	5'-CTAGTCATTACAATGGTGTCACCGGATAC GGCGCCGTTGGTTGCCTCGATGCCC-3' (SEQ ID NO:18)
$N^{134}A$ linker:	5'-GTACACGCATGCGGCGAGCAGCCCGG GA-3' (SEQ ID NO:19)

and 5'-CCGGTCCCGGGCTGCTCGCCGCATGC GT-3'
(SEQ ID NO:20)

pBRL-A was constructed by substituting the *Escherichia coli* maltose binding protein (MBP) and the *Bacillus circulans* chitin binding domain (CBD) coding regions in pMRB9GA with the CBD and the T4 DNA ligase coding regions, respectively, subcloned from the pBYT4 plasmid.

EXAMPLE II

Generating a thioester-tagged protein:

The pMRB10G construct from Example I contains the *Mth* RIR1 intein engineered to undergo thiol reagent induced cleavage at the N-terminal splice junction (Figure 1, N-terminal cleavage) and was used to isolate proteins with a C-terminal thioester as described previously for the *Sce* VMA and *Mxe* GyrA inteins (Chong et al. *supra* 1997); Evans et al., supra (1998)). Briefly, ER2566 cells (Evans et.al. (1998)) containing the appropriate plasmid were grown at 37°C in LB broth containing 100 µg/mL ampicillin to an OD_{600} of 0.5-0.6 followed by induction with IPTG (0.5 mM). Induction was either overnight at 15°C or for 3 hours at 30°C.

The cells were pelleted by centrifugation at 3,000xg for 30 minutes followed by resuspension in buffer A (20 mM Tris-HCl, pH 7.5 containing 500 mM NaCl). The cell contents were released by sonication. Cell debris was removed by

centrifugation at 23,000xg for 30 minutes and the supernatant was applied to a column packed with chitin resin (10 mL bed volume) equilibrated in buffer A. Unbound protein was washed from the column with 10 column volumes of buffer A.

Thiol reagent-induced cleavage was initiated by rapidly equilibrating the chitin resin in buffer B (20 mM Tris-HCl, pH 8 containing 500 mM NaCl and 100 mM 2-mercaptoethane-sulfonic acid (MESNA)). The cleavage reaction, which simultaneously generates a C-terminal thioester on the target protein, proceeded overnight at 4°C after which the protein was eluted from the column. The use of the pMRB10G construct resulted in the isolation of MBP with a C-terminal thioester (Figure2A).

Isolating proteins with an N-terminal cysteine

The pBRL-A construct from Example I contains an *Mth* RIR1 intein engineered to undergo controllable cleavage at its C-terminus, and was used to purify proteins with an N-terminal cysteine (Figure 1, C-terminal cleavage). The expression and purification protocol was performed as described in Example II, except with buffer A replaced by buffer C (20 mM Tris-HCl, pH 8.5 containing 500 mM NaCl) and buffer B replaced by buffer D (20 mM Tris-HCl, pH 7.0 containing 500 mM NaCl). Also, following equilibration of the

column in buffer D the cleavage reaction proceeded overnight at room temperature.

The expression of plasmid pBRL-A resulted in the purification of 4-6 mg/L cell culture of T4 DNA ligase possessing an N-terminal cysteine (Figure 2A). Protein concentrations were determined using the Bio-Rad protein assay (Bio-Rad Laboratories, Inc., Hercules, CA).

EXAMPLE III

Protein-protein ligation using Intein-mediated Protein Ligation

Intein-mediated protein ligation (IPL) was used to fuse two proteins (Figure 2B). Freshly isolated thioester-tagged protein from Example II was mixed with freshly isolated protein containing an N-terminal cysteine residue from Example II, with typical starting concentrations of 1-200 μM. The solution was concentrated with a Centriprep 3 or Centriprep 30 apparatus (Millipore Corporation, Bedford, MA) then with a Centricon 3 or Centricon 10 apparatus to a final concentration of 0.15-1.2 mM for each protein.

Ligation reactions proceeded overnight at 4°C and were visualized using SDS-PAGE with 12% Tris-glycine gels (Novex Experimental Technology, San Diego, CA) stained with Coomassie Brilliant Blue. Typical ligation efficiencies ranged from 20-60%.

Confirmation of ligation in IPL reactions

A Factor Xa site in MBP that exists 5 amino acids N-terminal from the site of fusion (Maina et al, *supra* (1988)) allowed amino acid sequencing through the ligation junction. The sequence obtained was NH_2-TLEGCGEQPTGXLK-COOH (SEQ ID NO:21) which matched the last 4 residues of MBP (TLEG) followed by a linker sequence (CGEQPTG (SEQ ID NO:22)) and the start of T4 DNA ligase (ILK). During amino acid sequencing, the cycle expected to yield an isoleucine did not have a strong enough signal to assign it to a specific residue, so it was represented as an X. The cysteine was identified as the acrylamide alkylation product.

The Factor Xa proteolysis was performed on 2 mg of ligation reaction involving MBP and T4 DNA ligase. This reaction mixture was bound to 3 mL of amylose resin (New England Biolabs, Inc., Beverly, MA) equilibrated in buffer A (see Example II). Unreacted T4 DNA ligase was rinsed from the column with 10 column volumes of buffer A. Unligated MBP and the MBP-T4 DNA ligase fusion protein were eluted from the amylose resin using buffer E (20 mM Tris-HCl, pH 7.5 containing 500 mM NaCl and 10 mM maltose). Overnight incubation of the eluted protein with a 200:1 protein:bovine Factor Xa (NEB) ratio (w/w) at 4°C resulted in the proteolysis of the fusion protein and regeneration of a band on SDS-PAGE gels that ran at a molecular weight similar to T4 DNA ligase.

N-terminal amino acid sequencing of the proteolyzed fusion protein was performed on a Procise 494 protein sequencer (PE Applied Biosystems, Foster City, CA).

Temperature sensitivity of the *Mth* RIR1 intein

The cleavage and/or splicing activity of the *Mth* RIR1 intein was more proficient when protein synthesis was induced at 15°C than when the induction temperature was raised to 37°C (Figure 3). The effect temperature has on the *Mth* RIR1 represents a way to control the activity of this intein for use in controlled splicing or cleavage reactions. Replacement of Pro^{-1} with a Gly and Cys^{1} with a Ser resulted in a double mutant, the pMRB9GS construct, which showed only *in vivo* C-terminal cleavage activity when protein synthesis was induced at 15°C but not at 37°C. Another double mutant, the pMRB9GA construct, displayed slow cleavage, even at 15°C, which allowed the accumulation of substantial amounts of the precursor protein and showed potential for use as a C-terminal cleavage construct for protein purification.

WHAT IS CLAIMED IS:

1. A method for fusion of expressed proteins, said method comprising the steps of:

(a) generating at least one C-terminal thioester-tagged first target protein;

(b) generating at least one second target protein having a specified N-terminal; and

(c) ligating said first and said second target proteins.

2. The method of claim 1, wherein said first target protein of step (a) is generated from a first plasmid comprising at least one first intein having N-terminal cleavage activity and said second target protein of step (b) is generated from a second plasmid comprising at least one second intein having C-terminal cleavage activity.

3. The method of claim 2, wherein said first intein comprises a first modified *Mth* RIR1 intein and wherein said second modified intein comprises a second modified *Mth* RIR1 intein.

4. The method of claim 3, wherein said first modified *Mth* RIR1 intein is selected from the group consisting of a Pro^{-1} to Ala mutant intein, a Pro^{-1} to Gly mutant intein, and a Pro^{-1} - Asn^{134} to Gly-Ala mutant intein, and wherein said second modified *Mth* RIR1 intein is selected from the group consisting of a Pro^{-1} - Cys^{1} to

Gly-Ser mutant intein and a Pro^{-1} - Cys^{1} to Gly-Ala mutant intein.

5. The method of claim 3, wherein said first plasmid is selected from the group consisting of pMRB8A, pMRB8G1 and pMRB10G, and wherein said second plasmid is selected from the group consisting of pMRB9GS, pMRB9GA and pBRL-A.

6. The method of claim 3, wherein said first target protein of step (a) is generated by thiol reagent-induced cleavage of said first modified *Mth* RIR1 intein and said second target protein of step (b) is generated by temperature and/or pH induced cleavage of said second modified *Mth* RIR1 intein.

7. The method of claim 2, wherein said specified N-terminal of step (b) comprises cysteine.

8. A method for fusion of expressed proteins, said method comprising the steps of:

(a) constructing a first plasmid comprising at least one first target protein and at least one first modified intein, wherein said first modified intein is capable of thiol reagent-induced cleavage to produce a thioester at the C-terminal of said first target protein;

(b) constructing a second plasmid comprising at least one second target protein and at least one second intein having C-terminal cleavage activity, wherein said second intein is capable of cleavage to produce a said second target protein having a specified N-terminal;

(c) generating at least one C-terminal thioester-tagged first target protein from said first plasmid of step (a);

(d) generating at least one second target protein having a specified N-terminal from said second plasmid of step (b); and

(e) ligating said first target protein of step (c) with said second target protein of step (d).

9. The method of claim 8, wherein step (c) further comprises purifying said C-terminal thioester-tagged first protein and step (d) further comprises purifying said second target protein having a specified N-terminal.

10. The method of claim 9, wherein said purifications of step (c) and step (d) comprise purification on a chitin resin column.

11. The method of claim 8, wherein said first intein of step (a) comprises a first modified *Mth* RIR1 intein, and wherein said second intein of step (b) comprises a second modified *Mth* RIR1 intein.

12. The method of claim 11, wherein said first modified *Mth* RIR1 intein is selected from the group consisting of a Pro^{-1} to Ala mutant intein, a Pro^{-1} to Gly mutant intein, and a Pro^{-1} - Asn^{134} to Gly-Ala mutant intein, and wherein said second modified *Mth* RIR1 intein is selected from the group consisting of a Pro^{-1} - Cys^{1} to Gly-Ser mutant intein and a Pro^{-1} - Cys^{1} to Gly-Ala mutant intein.

13. The method of claim 12, wherein said first plasmid of step (a) is selected from the group consisting of pMRB8A, pMRB8G1 and pMRB10G, and wherein said second plasmid of step (b) is selected from the group consisting of pMRB9GS, pMRB9GA and pBRL-A.

14. The method of claim 8, wherein said specified N-terminal comprises cysteine.

15. A fusion protein produced by the method of any one of claims 1-14.

16. A method for cyclic fusion of an expressed protein, said method comprising the steps of:

(a) constructing a plasmid comprising at least one target protein, at least one first intein having N-terminal cleavage activity, and at least one second

intein having C-terminal cleavage activity, wherein said first intein is capable of thiol reagent-induced cleavage to produce a thioester at the C-terminal of said target protein and wherein said second intein is capable of cleavage to produce a specified amino acid at the N-terminal of said target protein;

(b) generating a C-terminal thioester-tagged target protein having a specified amino acid at its N-terminal from the plasmid of step (a); and

(c) ligating the N-terminus of said target protein to the C-terminus of said target protein to produce a cyclic protein.

17. A method for polymerization of an expressed protein, said method comprising the steps of:

(a) constructing a plasmid comprising at least one target protein, at least one first intein having N-terminal cleavage activity, and at least one second intein having C-terminal cleavage activity, wherein said first intein is capable of thiol reagent-induced cleavage to produce a thioester at the C-terminal of said target protein and wherein said second intein is capable of cleavage to produce a specified amino acid at the N-terminal of said target protein;

(b) generating a C-terminal thioester-tagged target protein having a specified amino acid at its N-terminal from the plasmid of step (a); and

(c) intermolecular ligation of said target proteins to yield a protein polymer.

18. The method of claim 16 or 17, wherein said first intein of step (a) comprises a first modified *Mth* RIR1 intein, and wherein said second intein of step (a) comprises a second modified *Mth* RIR1 intein.

19. The method of claim 18, wherein said first modified *Mth* RIR1 intein is selected from the group consisting of a Pro^{-1} to Ala mutant intein, a Pro^{-1} to Gly mutant intein, and a Pro^{-1} - Asn^{134} to Gly-Ala mutant intein, and wherein said second modified *Mth* RIR1 intein is selected from the group consisting of a Pro^{-1} - Cys^{1} to Gly-Ser mutant intein and a Pro^{-1} - Cys^{1} to Gly-Ala mutant intein.

20. The method of claim 16 or 17, wherein said specified amino acid comprises cysteine.

21. A cyclic protein produced by the method of any one of claim 16.

22. A modified intein comprising a mutant *Mth* RIR1 intein capable of thiol reagent-induced cleavage to produce a thioester at the C-terminal of an adjacent target protein.

23. The modified intein of claim 22, wherein said mutant *Mth* RIR1 intein is selected from the group consisting of a Pro^{-1} to Ala mutant intein, a Pro^{-1} to Gly mutant intein, and a Pro^{-1} - Asn^{134} to Gly-Ala mutant intein.

24. A modified intein comprising a mutant intein capable of pH and temperature-induced cleavage to produce a specified residue at the N-terminal of an adjacent target protein.

25. The modified intein of claim 24, wherein said mutant intein comprises a mutant *Mth* R1R1 intein.

26. The modified intein of claim 25, wherein said specified residue is cysteine.

27. The modified intein of claim 25, wherein said mutant *Mth* R1R1 intein is selected from the group consisting of a Pro^{-1} - Cys^{1} to Gly-Ser mutant intein and a Pro^{-1} - Cys^{1} to Gly-Ala mutant intein.

28. A plasmid comprising at least one modified intein of any one of claims 22-27.

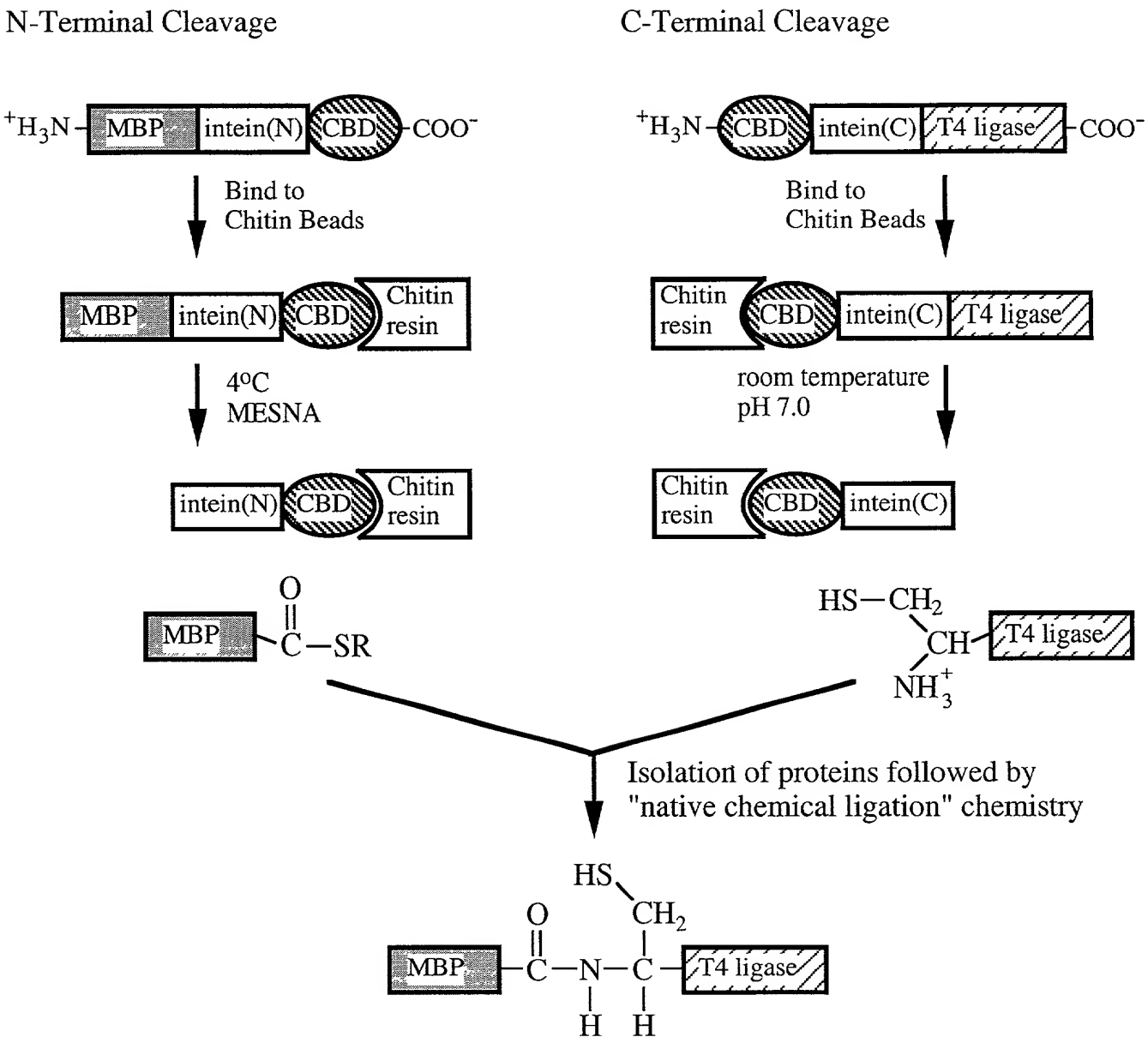
29. A plasmid comprising a modified *Mth* RIR1 intein, wherein said plasmid is selected from the group consisting of pMRB8P, pMRB8A, pMRB8G1, pMRB9GS, pMRB9GA, pMRB10G and pBRL-A.

30. A DNA segment encoding a modified *Mth* RIR1 intein, wherein said DNA segment is obtainable from a plasmid selected from the group consisting of pMRB8P, pMRB8A, pMRB8G1, pMRB9GS, pMRB9GA, pMRB10G and pBRL-A.

ABSTRACT

A method for the ligation of expressed proteins which utilizes inteins, for example the RIR1 intein from *Methanobacterium thermotrophicum*, is provided. Constructs of the *Mth* RIR1 intein in which either the C-terminal asparagine or N-terminal cysteine of the intein are replaced with alanine enable the facile isolation of a protein with a specified N-terminal, for example, cysteine for use in the fusion of two or more expressed proteins. The method involves the steps of generating a C-terminal thioester-tagged target protein and a second target protein having a specified N-terminal via inteins, such as the modified *Mth* RIR1 intein, and ligating these proteins. A similar method for producing a cyclic or polymerized protein is provided. Modified inteins engineered to cleave at their C-terminus or N-terminus, respectively, and DNA and plasmids encoding these modified inteins are also provided.

FIGURE 1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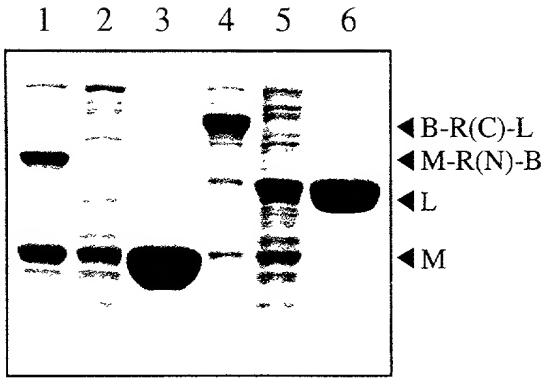

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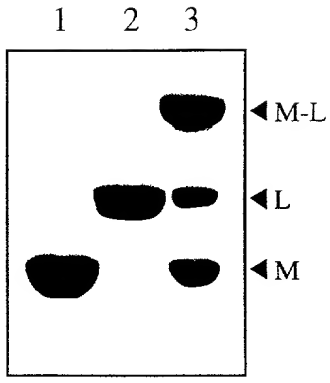

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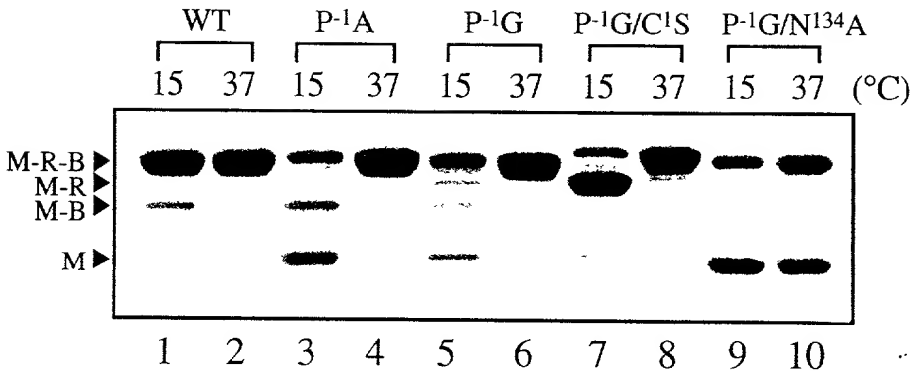
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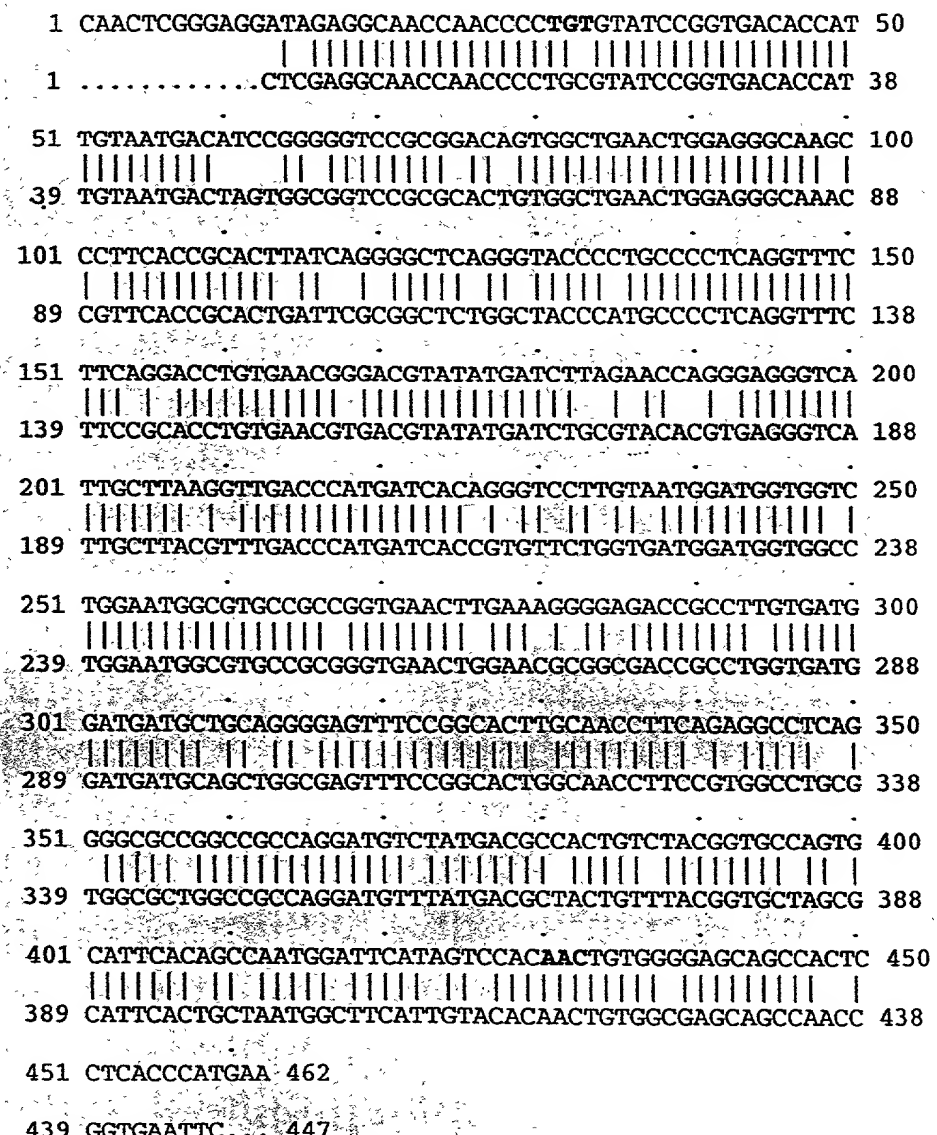
Figure 4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<211> 447

<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Chemically
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thermoautotrophicum.

<400> 24
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ggcgagtttc cggcactggc aaccttccgt ggcctgcgtg gcgctggccg ccaggatgtt 360
tatgacgcta ctgtttacgg tgctagcgca ttcactgcta atggcttcat tgtacacaac 420
tgtggcgagc agccaaccgg tgaattc 447

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DECLARATION
AND POWER OF ATTORNEY
Original Application

Attorney Docket No. NEB-154

As a below named inventor, I hereby declare that:
My residence, post address and citizenship are as stated below next to my name
I believe that I am the original, first and sole inventor (in only one name is listed at 201 below) or an original, first and joint inventor (if plural names are listed at 201-203 below) of the subject matter which is claimed and which a patent is sought on the invention entitled:
INTEIN-MEDIATED PROTEIN LIGATION OF EXPRESSED PROTEINS

which is described and claimed in:
[X] the attached specification or [ ] the specification in Application Serial No. _____ filed_____
(for declaration not accompanying application)
And was amended on_____
if applicable

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendments referred to above. I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, §1.56(a).
I hereby claim foreign priority benefits under Title 35, United States Code, §119 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

FOREIGN APPLICATION(S) IF ANY, FILED WITHIN 12 MONTHS PRIOR TO THE FILING DATE OF THIS APPLICATION			
COUNTRY	APPLICATION	DATE OF FILING (day, month, year)	PRIORITY CLAIMED UNDER 35 U.S.C. 119
			YES NO
			YES NO

ALL FOREIGN APPLICATION(S) IF ANY, FILED MORE THAN 12 MONTHS PRIOR TO THE FILING DATE OF THIS APPLICATION			
COUNTRY	APPLICATION	(day, month, year)	PRIORITY CLAIMED UNDER 35 U.S.C. 119

I hereby claim the benefit under Title 35, United States Code §120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code §112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, §1.56(a) which occurred between the filing date of the prior application and the national or PCT international filing date of this application:

Application Serial No.	Filing Date	Status (Patented, Pending, Abandoned)
08/811,492	5-Mar-97	Patented
60/102,413	30-Sep-98	Pending

POWER OF ATTORNEY:

As a named inventor, I hereby appoint the following attorney with full powers of association, substitution and revocation to prosecute this application and transact all business in the Patent and Trademark Office connected therewith:

Gregory D. Williams
(Registration No. 30901)

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207	Full Name of Inventor	Last Name	First Name	Middle Name
	Residence & Citizenship	City	State/Foreign Country	Citizenship
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208	Full Name of Inventor	Last Name	First Name	Middle Name
	Residence & Citizenship	City	State/Foreign Country	Citizenship
	Post Office Address	Post Office Address	City/State/Country	Zip Code
209	Full Name of Inventor	Last Name	First Name	Middle Name
	Residence & Citizenship	City	State/Foreign Country	Citizenship
	Post Office Address	Post Office Address	City/State/Country	Zip Code

I hereby further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true and further that these statements were made with the knowledge that willful statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Signature of Inventor 201 Thomas C. Evans	Date 2/12/99
Signature of Inventor 202	Date 2/12/99
Signature of Inventor 203	Date
Signature of Inventor 204	Date
Signature of Inventor 205	Date
Signature of Inventor 206	Date
Signature of Inventor 207	Date
Signature of Inventor 208	Date
Signature of Inventor 209	Date